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Phosphorylation of the Protein Kinase Mutated in Peutz-Jeghers Cancer Syndrome, LKB1/STK11, at Ser^{431} by p90^{RSK} and cAMP-dependent Protein Kinase, but Not Its Farnesylation at Cys^{433}, Is Essential for LKB1 to Suppress Cell Growth*

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Peutz-Jeghers syndrome is an inherited cancer syndrome that results in a greatly increased risk of developing tumors in those affected. The causative gene is a protein kinase termed LKB1, predicted to function as a tumor suppressor. The mechanism by which LKB1 is regulated in cells is not known. Here, we demonstrate that stimulation of Rat-2 or embryonic stem cells with activators of ERK1/2 or of cAMP-dependent protein kinase induced phosphorylation of endogenously expressed LKB1 at Ser^{431}. We present pharmacological and genetic evidence that p90^{RSK} mediated this phosphorylation in response to agonists that activate ERK1/2 and that cAMP-dependent protein kinase mediated this phosphorylation in response to agonists that activate adenylate cyclase. Ser^{431} of LKB1 lies adjacent to a putative prenylation motif, and we demonstrate that full-length LKB1 expressed in 293 cells was prenylated by addition of a farnesyl group to Cys^{433}. Our data suggest that phosphorylation of LKB1 at Ser^{431} does not affect farnesylation and that farnesylation does not affect phosphorylation at Ser^{431}. Phosphorylation of LKB1 at Ser^{431} did not alter the activity of LKB1 to phosphorylate itself or the tumor suppressor protein p53 or alter the amount of LKB1 associated with cell membranes. The reintroduction of wild-type LKB1 into a cancer cell line that lacks LKB1 suppressed growth, but mutants of LKB1 in which Ser^{431} was mutated to Ala to prevent phosphorylation of LKB1 were ineffective in inhibiting growth. In contrast, a mutant of LKB1 that cannot be prenylated was still able to suppress the growth of cells.

Peutz-Jeghers syndrome is an autosomal dominantly inherited disorder that predisposes to a wide spectrum of benign and malignant tumors (1, 2). It is caused by mutation of a widely expressed protein kinase of unknown function termed LKB1 (also known as STK11) (3, 4). To date, over 60 different mutations have been mapped to LKB1, many of which would be expected to impair LKB1 activity. These discoveries have aroused great interest because they indicate that LKB1 is likely to function in cells as a tumor suppressor, and consistent with this, overexpression of LKB1 in a number of tumor cell lines has been shown to suppress cell growth by inducing a G_1 cell cycle block (5). However, little is known regarding the mechanism by which LKB1 activity is regulated in cells, and no substrates for LKB1 have thus far been identified.

LKB1 is a 436-amino acid protein possessing a kinase domain (residues 50–337) that is only distantly related to other mammalian kinases. The N-terminal non-catalytic domain comprises both a nuclear localization signal (6) and a putative cytoplasmic retention signal (7). There are no yeast homologs of LKB1, but there are putative homologs in *Xenopus* (termed XEEK1, with 84% overall identity to LKB1) (8) and *Caenorhabditis elegans* (termed PAR-4, with 26% overall identity to LKB1 and 41% identity in the kinase domain) (9). In *Drosophila*, an uncharacterized protein kinase listed in the NCBI Protein Database (NCBI accession number AAF54972) possesses 44% overall identity to LKB1.

Recently, a C-terminal fragment of LKB1 was shown to be phosphorylated at Ser^{431} by the cAMP-dependent protein kinase (10). Ser^{431} of LKB1 lies in the sequence Lys-Xaa-Arg-Arg-Xaa-Ser (where Xaa is any amino acid), which is conserved in all known mammalian LKB1 sequences and in *Xenopus* XEEK1. This study did not establish whether full-length or endogenously expressed LKB1 was phosphorylated at Ser^{431} in response to stimuli that activated cAMP-dependent protein kinase (PKA)[1] or the role that this phosphorylation played in enabling LKB1 to suppress cell growth. Ser^{431} lies in the consensus sequence for phosphorylation by a group of kinases related to PKA, *viz.* p90 ribosomal S6 kinase (p90^{RSK}), mitogen- and stress-stimulated protein kinase (MSK1), and p70 ribosomal S6 kinase (S6K1) (11–13), that collectively belong to the AGC kinase subfamily. p90^{RSK} is activated in cells by growth factors and phorbol esters and by ERK1/2 MAPK family members (14), whereas MSK1 is activated *in vivo* by two dif-

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; p90^{RSK}, p90 ribosomal S6 kinase; MSK1, mitogen- and stress-activated protein kinase-1; S6K1, p70 ribosomal S6 kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; ES, embryonic stem; EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; CREB, cAMP response element-binding protein; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; PDK1, 3-phosphoinositide-dependent protein kinase; GSK3, glycogen synthase kinase-3; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PKB, protein kinase B; AGC, protein kinases similar to PKA, PKG, and PKC.

ferent types of MAPK family members, *viz.* ERK1/2 and the stress- and cytokine-activated p38 MAPK (13). S6K1 is activated *in vivo* by growth factors through a phosphatidylinositol 3-kinase-dependent pathway and by phorbol esters and certain cellular stresses through a phosphatidylinositol 3-kinase-independent pathway (15).

Ser^{431} is located 6 amino acids from the C terminus of LKB1, and the residues that follow Ser^{431} in human LKB1 are Ala-Cys-Lys-Gln-Gln. The cysteine 2 residues C-terminal from Ser^{431} (Cys^{433}) thus lies 4 residues from the end of the protein in a consensus sequence known as the *CAAX* motif, which mediates the prenylation of many proteins (16, 17). This sequence including the Cys residue is conserved in C-terminal sequences of mammalian, *Xenopus*, and *Drosophila* LKB1. Uhler and co-workers (10) have recently demonstrated that a C-terminal fragment of LKB1, when overexpressed in 293 cells, was prenylated at Cys^{433}. However, these authors did not establish whether full-length LKB1 was prenylated by addition of a geranylgeranyl (C_{20}) or a farnesyl (C_{15}) moiety to Cys^{433} or if prenylation of LKB1 was required for LKB1 to suppress the growth of cells.

Here, we report that p90^{RSK}, MSK1, S6K1, and PKA phosphorylate full-length LKB1 specifically at Ser^{431} *in vitro*. We show that agonists that activate these kinases in Rat-2 cells and embryonic stem (ES) cells induce the phosphorylation of endogenous LKB1 at Ser^{431}. We use signal transduction inhibitors and ES cells lacking p90^{RSK} activity and ES cells deficient in MSK1 to demonstrate that phosphorylation of LKB1 induced by EGF and TPA is likely to be mediated by p90^{RSK} rather than by MSK1 or S6K1 and that phosphorylation of LKB1 induced by forskolin is mediated by PKA. We show that full-length LKB1 expressed in 293 cells is prenylated by addition of a farnesyl moiety at Cys^{433}, and we provide evidence that phosphorylation of LKB1 at Ser^{431}, but not its farnesylation at Cys^{433}, is likely to be important in mediating the ability of LKB1 to suppress cell growth.

EXPERIMENTAL PROCEDURES

Materials—Protease inhibitor mixture tablets, histone 2B, Fugene-6 transfection reagent, and G418 were from Roche Molecular Biochemicals. PD 184352 was from Upstate Biotechnology. U0126 was from Promega. Rapamycin, H-89, Ro 318220, PD 98059, and zwittergent 3–16 were from Calbiochem. EGF, insulin-like growth factor-1, microcystin-LR, dialyzed fetal bovine serum, and other tissue culture reagents were from Life Technologies, Inc. (*R*)-[2-^{14}C]Mevalonic acid lactone and ^{32}P-labeled inorganic phosphate was from Amersham Pharmacia Biotech. Forskolin, TPA, mevastatin, and dimethyl pimelimidate were from Sigma. The pre-cast BisTris/SDS-4–12% gradient polyacrylamide gels were from Invitrogen. All peptides used in this study were synthesized by Dr. G. Blomberg (University of Bristol, Bristol, United Kingdom). CREB (13) and BAD (18) were expressed as GST fusion proteins in *Escherichia coli* as described previously. Mouse p53 expressed in bacteria was prepared as described previously (19).

Antibodies—Antibodies recognizing LKB1 were raised in sheep against peptide GELMSVGMDTFIHRID (corresponding to residues 15–30 of mouse LKB1), and the GST-LKB1 protein expressed in *E. coli*. The antibodies were affinity-purified on CH-Sepharose covalently coupled to the antigens used to raise the antibodies. The antibody raised against GST-LKB1 was also passed through a column of CH-Sepharose coupled to GST, and the antibody that did not bind was selected. The phospho-specific antibody recognizing LKB1 phosphorylated at Ser^{431} (termed antibody S431-P) was raised in sheep against peptide SNKIRRLSACKQQ (corresponding to the residues 424–436 of mouse LKB1; the underlined residue is phosphoserine). The antibody was affinity-purified on CH-Sepharose covalently coupled to the phosphorylated peptide and then passed through a column of CH-Sepharose coupled to the non-phosphorylated peptide. Antibody that did not bind to the latter column was selected. The antibodies raised against GST-LKB1 and antibody S431-P are available from Upstate Biotechnology, Inc. Antibodies that recognize S6K1 were raised against peptide AGVFDIDLDQPEDAGSEDEL (corresponding to residues 1–20 of human S6K1). Antibodies that recognize isoforms of p90^{RSK} were raised against peptide RNQSPVLEPVGRSTLAQRRGIKK (residues 712–734 of human p90^{RSK2}). Antibodies that recognize MSK1 were raised against peptide FKRNAAVIDPLQFHMGVER (corresponding to residues 384–402 of MSK1) (13). Antibodies recognizing ERK1 and ERK2, phospho-specific antibodies recognizing the activated forms of ERK1 and ERK2, and phospho-specific antibodies recognizing GSK3α phosphorylated at Ser^{21} and GSK3β phosphorylated at Ser^{9} were from New England Biolabs Inc. Antibodies recognizing CREB, phospho-specific antibodies recognizing CREB phosphorylated at Ser^{133}, and the antibodies used for immunoblotting Ras were from Upstate Biotechnology, Inc. The antibodies used for immunoprecipitating Ras were a generous gift from R. Marais (Institute for Cancer Research). Secondary antibodies coupled to horseradish peroxidase used for immunoblotting were from Pierce, and monoclonal antibodies recognizing GST and FLAG epitope tags were from Sigma.

General Methods and Buffers—Phosphoamino acid analysis of ^{32}P-labeled peptides, restriction enzyme digests, DNA ligations, site-directed mutagenesis, and other recombinant DNA procedures were performed using standard protocols. All DNA constructs were verified by DNA sequencing. This was performed by the Sequencing Service at the School of Life Sciences of the University of Dundee using DYEnamic ET terminator chemistry (Amersham Pharmacia Biotech) on Applied Biosystems automated DNA sequencers. Buffer A contained 50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.27 M sucrose, and 0.1% (by volume) 2-mercaptoethanol. Buffer B contained 50 mM Tris-HCl (pH 7.5) and 0.1 mM EGTA. SDS-sample buffer contained 50 mM Tris-HCl (pH 6.8), 2% (by mass) SDS, 10% (by volume) glycerol, and 1% (by volume) 2-mercaptoethanol. Buffer C contained 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1% (by mass) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR, 0.1% (by volume) 2-mercaptoethanol, and Complete proteinase inhibitor mixture (one tablet/25 ml). Buffer D contained 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR, 0.1% (by volume) 2-mercaptoethanol, and Complete proteinase inhibitor mixture (one tablet/25 ml).

Cloning of Mouse LKB1—A polymerase chain reaction-based strategy was used to prepare an N-terminal FLAG epitope-tagged cDNA construct encoding mouse LKB1 using, as a template, an expressed sequence tag encoding full-length mouse LKB1 (NCBI accession number AA542163, IMAGE number 550355) obtained from the IMAGE consortium (20). The construct was obtained using the 5′-primer atgcatactagtgccaccatggactactacaaggacgacgatgacaaggacgtggcggaccccgagccgttggg and the 3′-primer gacagaactagttcactgctgcttgcaggccgaga. The resulting polymerase chain reaction fragment was cloned into the pCR-Topo2.1 vector (Invitrogen) and subsequently subcloned as an *Eco*RI-*Eco*RI fragment into the pCMV5 vector (to encode expression of FLAG-LKB1 in mammalian cells) (21) and as a *Spe*I-*Spe*I fragment into the pEBG-2T vector (to encode for expression of GST-FLAG-LKB1 in mammalian cells) (22) and as an *Eco*RI-*Eco*RI fragment into the pGEX-4T-1 vector (to encode for expression of GST-FLAG-LKB1 in *E. coli*). The indicated site-directed mutagenesis was performed using the QuickChange kit (Stratagene). To prepare the catalytically inactive mutant of LKB1 termed LKB1(KD) (where KD is kinase-dead), Asp^{194} in subdomain VII of the kinase domain was mutated to Ala.

Expression of GST-LKB1 in E. coli—The pGEX-4T-1 constructs encoding GST-LKB1 or the indicated mutants of LKB1 were transformed into *E. coli* BL21 cells, and a 0.5-liter culture was grown at 37 °C in Luria broth containing 100 μg/ml ampicillin until the absorbance at 600 nm was 0.6. Isopropyl-β-D-galactosidase (250 μM) was added, and the cells were cultured for a further 16 h at 26 °C. The cells were resuspended in 25 ml of ice-cold Buffer C and lysed by one round of freeze/thawing, and the lysates were sonicated to fragment the DNA. The lysates were centrifuged at 4 °C for 30 min at 20,000 × *g*, and the supernatant was filtered through a 0.44-μm filter and incubated for 60 min on a rotating platform with 1 ml of glutathione-Sepharose previously equilibrated in Buffer C. The suspension was centrifuged for 1 min at 3000 × *g*, and the beads washed three times with 15 ml of Buffer C containing 0.5 M NaCl and then a further 10 times with 15 ml of Buffer A. The protein was eluted from the resin at ambient temperature by incubation with 2 ml of Buffer A containing 20 mM glutathione, and the beads were removed by filtration through a 0.44-μm filter. The eluate was divided into aliquots, snap-frozen in liquid nitrogen, and stored at −80 °C.

Expression of GST-LKB1 in Human Embryonic Kidney 293 Cells—To express GST-LKB1 or the indicated mutants of LKB1 in human embryonic kidney 293 cells, 20 dishes (10-cm diameter) of 293 cells were cultured, and each dish was transfected with 8 μg of the pEBG-2T

construct using a modified calcium phosphate method (23). 36 h post-transfection, the cells were lysed in 1 ml of ice-cold Buffer C; the lysates were pooled and centrifuged at 4 °C for 10 min at 13,000 × *g*; and the GST fusion proteins were purified by affinity chromatography on glutathione-Sepharose and stored as described above.

Cell Culture, Stimulation, and Cell Lysis—The rat embryonic fibroblast cell line Rat-2 and human G361 malignant melanoma cells were obtained from European Tissue Culture Collection. Rat-2 cells were cultured on 15-cm diameter dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. G361 cells were cultured on 10-cm diameter dishes in McCoy's 5a medium supplemented with 2 mM glutamine and 10% (by volume) fetal calf serum. The MSK1$^{+/+}$ and MSK1$^{-/-}$ ES cells (24) and the PDK1$^{+/+}$ and PDK1$^{-/-}$ ES cells (25) were cultured on gelatinized 15-cm diameter dishes in KnockOut™ DMEM supplemented with 10% KnockOut™ serum replacement, 0.1 mM nonessential amino acids, antibiotics (100 units of penicillin G and 100 mg/ml streptomycin), 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 1000 units/ml ESGRO™ (murine leukemia inhibitory factor) to prevent differentiation of the cells. Prior to stimulation, Rat-2 cells were cultured in the absence of serum overnight, whereas the ES cell lines were deprived of serum for 4 h. Inhibitors were dissolved in Me_2SO at a 1000-fold higher concentration than they were used. These inhibitors or the equivalent volume of Me_2SO as a control was added to the tissue culture medium 30 min prior to stimulation unless indicated otherwise. The cells were stimulated with the indicated agonists, lysed in 1 ml of ice-cold Buffer C, and centrifuged at 4 °C for 5 min at 16,000 × *g*. The supernatants were frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were determined using the Bradford method (58), and bovine serum albumin was employed as the standard.

Phosphorylation of LKB1 by AGC Kinases—PKA was purified from bovine heart by Dr. C. MacKintosh in the Medical Research Council Unit. MSK1 and p90^{RSK1} expressed as GST fusion proteins were purified from TPA-stimulated 293 cells (13). Hexahistidine-tagged S6K1, which lacks the carboxyl-terminal 104 residues and in which Thr^{412} is mutated to Glu, was expressed in insect cells and activated *in vitro* by phosphorylation with PDK1 (26). GST-LKB1(KD), GST-LKB1(S431A), GST-CREB, GST-BAD, or histone 2B (all at 1 μg) and the peptide Crosstide (GRPRTSSFAEG, 30 μM) or Kemptide (LRRASLG, 30 μM) were incubated in a total volume of 40 μl at 30 °C with 1 units/ml PKA, GST-MSK1, GST-p90^{RSK1}, and His-S6K1 in Buffer B containing 10 mM magnesium acetate, 100 μM [γ-^{32}P]ATP (1000 cpm/pmol), and 1 μM microcystin-LR. After incubation for 15 min, incorporation of phosphate into peptides was determined using phosphocellulose P-81 paper (27), and the incorporation of phosphate into LKB1, CREB, BAD, and histone 2B was determined following the electrophoresis of samples on BisTris-4–12% polyacrylamide gel electrophoresis gels and autoradiography of the gels.

Mapping the Site on LKB1 Labeled by PKA, p90^{RSK1}, S6K1, and MSK1—To map the site on LKB1 phosphorylated by PKA, p90^{RSK1}, S6K1, and MSK1, GST-LKB1(KD) was phosphorylated by these kinases as described above, except that the reaction was performed for 60 min, and a 10-fold higher specific activity of ATP was employed. The reactions were terminated by adding 1% (by mass) SDS and 10 mM dithiothreitol and heated at 100 °C for 1 min. After cooling, 4-vinylpyridine was added to a concentration of 1% (by volume), and the sample was left on a shaking platform for 30 min at room temperature to alkylate cysteine residues. The sample was subjected to electrophoresis on a BisTris-4–12% polyacrylamide gel electrophoresis gel, and the 82-kDa ^{32}P-labeled band corresponding to LKB1(KD) was excised and cut into smaller pieces. These were washed sequentially for 15 min on a vibrating platform with 1 ml of the following: water, a 1:1 mixture of water and acetonitrile, 0.1 M ammonium bicarbonate, a 1:1 mixture of 0.2 M ammonium bicarbonate and acetonitrile, and finally, acetonitrile. The gel pieces were dried by rotary evaporation and incubated in 0.3 ml of 50 mM ammonium bicarbonate and 0.05% (by mass) zwittergent 3–16 containing 2 μg of alkylated trypsin. After 16 h, the supernatant was removed; the gel pieces were washed for 10 min in a further 0.3 ml of 50 mM ammonium bicarbonate, 0.05% (by mass) zwittergent 3–16, and 0.1% (by volume) trifluoroacetic acid; and the combined supernatants containing >90% of the ^{32}P radioactivity were chromatographed on a Vydac 218TP54 C_{18} column (Separations Group, Hesperia, CA) as described in the legend to Fig. 1.

^{32}P Labeling of 293 Cells Transfected with LKB1—293 cells were transfected with a pCMV5-encoded DNA construct expressing either wild-type FLAG-LKB1 or the indicated LKB1 mutants. 36 h post-transfection, the cells were washed with phosphate-free DMEM, incubated for 3 h with [^{32}P]orthophosphate (1 mCi/ml), and then left unstimulated or stimulated with forskolin (20 μM) for 10 min. FLAG-LKB1 was immunoprecipitated from the cleared lysate with anti-FLAG antibodies (5 μg of antibody conjugated to 5 μl of protein G-Sepharose). The immunoprecipitates were washed 10 times with 1 ml of Buffer C containing 0.5 M NaCl and once with Buffer A. The immunoprecipitated protein was alkylated with 4-vinylpyridine and subjected to SDS-polyacrylamide gel electrophoresis; and following autoradiography, the ^{32}P-labeled band corresponding to FLAG-LKB1 was excised, digested with trypsin, and analyzed by chromatography on a C_{18} column as described above.

Phosphopeptide Sequence Analysis—Peptides were analyzed by MALDI-TOF mass spectrometry on a PerSeptive Biosystems Elite-STR mass spectrometer using α-cyanocinnamic acid as the matrix. Spectra were obtained in both the linear and reflector modes. The sequence identity of each peptide was also confirmed by Edman sequencing on an Applied Biosystems 476A sequencer, and the site of phosphorylation was determined by solid-phase Edman degradation of the peptide coupled to Sequelon-AA membrane (Milligen) as described previously (28).

Immunoprecipitation of LKB1—The polyclonal anti-LKB1 antibody raised against GST-LKB1 (1 mg) was covalently coupled to protein G-Sepharose (1 ml) using dimethyl pimelimidate (29). Rat-2 (0.5 mg) or ES (1 mg) cell lysates were incubated for 60 min at 4 °C with the LKB1-protein G-Sepharose conjugate (5 μl). The immunoprecipitates were washed twice with 1 ml of Buffer C containing 0.5 M NaCl and twice either with Buffer B for immunoblot analysis or with Buffer A for the p53 kinase assays. For immunoblot analysis, the beads were resuspended in SDS sample buffer that did not contain 2-mercaptoethanol.

Immunoblotting—For blots of total cell lysates, 20 μg of protein was used. For blots of LKB1 immunoprecipitation, 5 μl of beads that had been incubated with 0.5 mg of Rat-2 or 1 mg of ES cell lysate was used. Samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. For experiments in which LKB1 and GSK3α isoforms were being immunoblotted, the membranes were incubated in 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% (by volume) Tween, and 10% (by mass) skimmed milk for 7 h at 4 °C in the presence of 1 μg/ml antibody. Immunoblotting with antibody S431-P (1 μg/ml) was carried out as described above, except that non-phosphorylated peptide (10 μg/ml) corresponding to the antigen used to raise the antibody was included. For experiments using all other commercial antibodies, we used a 1000-fold dilution of the stock antibody and 5% (by mass) bovine serum albumin in place of skimmed milk. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Immunoprecipitation and Assay of p90^{RSK}, MSK1, and S6K1—The indicated amounts of Rat-2 cell lysate were used to immunoprecipitate MSK1 (500 μg of protein), p90^{RSK} (50 μg of protein), and S6K1 (100 μg of protein). The lysates were incubated at 4 °C for 1 h on a shaking platform with 5 μg of each antibody coupled to 5 μl of protein G-Sepharose. The immunoprecipitates were washed twice with 1 ml of Buffer C containing 0.5 M NaCl and twice with 1 ml of Buffer A. The assay (50 μl) contained washed protein G-Sepharose immunoprecipitate, 50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.1% (by volume) 2-mercaptoethanol, 2.5 μM protein kinase inhibitor (TTYADFIASGRTGRRNAIHD, peptide inhibitor of PKA), 10 mM magnesium acetate, 0.1 mM [γ-^{32}P]ATP (~1000 cpm/pmol), and Crosstide (GRPRTSSFAEG, 30 μM) (30). The assays were carried out for 15 min at 30 °C, with the assay tubes being agitated continuously to keep the immunoprecipitate in suspension, and then terminated and analyzed as described previously (27). 1 milliunit of activity is the amount of enzyme that catalyzes the phosphorylation of 1 pmol of Crosstide in 1 min.

LKB1 Autophosphorylation and Phosphorylation of p53—0.5 μg of GST-LKB1, GST-LKB1(KD), GST-LKB1(S431A), or GST-LKB1(S-431D) expressed in 293 cells or LKB1 that had been immunoprecipitated from Rat-2 cells was incubated in a 50-μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.1% β-mercaptoethanol, 0.1 mM EGTA, 10 mM manganese chloride, 0.5 μM microcystin-LR, and 100 μM [γ-^{32}P]ATP (1000 cpm/pmol) in the presence or absence of 2 μg of bacterially expressed mouse p53. After incubation for 60 min at 30 °C, the reactions were terminated by addition of SDS sample buffer, and the samples were electrophoresed on BisTris-4–12% polyacrylamide gel electrophoresis gels and analyzed by autoradiography.

Preparation of Cytosolic and Membrane Fractions—Rat-2 cells cultured on 10-cm diameter dishes were washed once with phosphate-buffered saline and then scraped into 2 ml of Buffer D. After incubation on ice for 5 min, the cells were lysed by passing them six times through a chamber containing a ball bearing, in which the space between the

chamber wall and the ball bearing was 0.014 mm. The homogenate was centrifuged twice at 1500 × *g* for 10 min to remove unbroken cells and nuclei, and the mitochondrial fraction was pelleted by centrifugation at 10,000 × *g* for 10 min. The supernatant was then centrifuged at 100,000 × *g* for 1 h to pellet the membrane fraction, and the supernatant was used as the cytosolic fraction. The membrane fraction was washed by resuspension in 2 ml of Buffer D containing 0.5 M NaCl, and the membranes were pelleted again by centrifugation at 100,000 × *g* for 1 h. The resulting membrane pellet was then solubilized in 0.5 ml of Buffer C containing Triton X-100 and centrifuged at 100,000 × *g* for 1 h to remove any insoluble debris. The supernatant was taken as the membrane fraction.

Labeling of 293 Cells with [^{14}C]Mevalonic Acid—293 cells were transfected with the indicated expression constructs expressing wild-type and mutant forms of FLAG-LKB1. 16 h post-transfection, the cells were washed twice in DMEM containing 10% (v/v) dialyzed fetal bovine serum and 25 μM mevastatin and incubated for 90 min at 37 °C. During this period, (*R*)-[2-^{14}C]mevalonic acid lactone was evaporated to dryness under a constant stream of nitrogen at 50 °C and converted to the sodium salt of mevalonic acid by incubation in 1 ml of 0.1 M NaOH for 1 h at 37 °C, and the mixture was then neutralized with 2 M HCl. The cells were washed twice with DMEM containing 10% (v/v) dialyzed fetal bovine serum and 25 μM mevastatin and then incubated in 5 ml of DMEM containing 10% (v/v) dialyzed fetal bovine serum, 25 μM mevastatin, and 2 μCi/ml (*R*)-[2-^{14}C]mevalonic acid lactone. After 20 h at 37 °C, the cells were lysed in Buffer C, and FLAG-LKB1 and Ras were immunoprecipitated from the cleared lysate with anti-FLAG or anti-Ras antibodies (5 μg of antibody conjugated to 5 μl of protein G-Sepharose). The immunoprecipitates were washed 10 times with 1 ml of Buffer C containing 0.5 M NaCl and once with Buffer A and then resuspended in SDS sample buffer. The samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and the ^{14}C-labeled proteins were detected using standard PhosphorImager analysis with a screen that detects ^{14}C radioactivity.

Growth Suppression of G361 Cells—G361 cells were cultured to 50% confluence on 10-cm diameter dishes and transfected with 2.5 μg of the indicated wild-type and mutant LKB1 in the pCMV5 vector together with 2.5 μg of the pCI-neo vector (Promega) using Fugene-6 transfection reagent following the manufacturer's protocol. A triplicate set of dishes was used for each condition. After 48 h, G418 was added to the medium to a final concentration of 3 mg/ml, and the medium was changed every 48 h, maintaining G418. After 16 days the cells were Giemsa-stained, and the average number of colonies present per cm^2 on each dish was counted.

RESULTS

Phosphorylation of LKB1 at Ser^{431} by Different AGC Kinases—To compare the phosphorylation of LKB1 by different AGC kinase members, we expressed a catalytically inactive point mutant of LKB1 in *E. coli* as a fusion protein with GST (hereafter termed GST-LKB1(KD)). PKA, p90^{RSK}, MSK1, and S6K1, but not PKB (all at ~1 unit/ml), phosphorylated GST-LKB1(KD) (Fig. 1*A*). Control experiments showed that, under the same conditions, the pro-apoptotic protein BAD was phosphorylated with similar efficiency by PKA, p90^{RSK}, PKB, and MSK1 (18), whereas as expected, the transcription factor CREB was phosphorylated to a similar extent by MSK1 and PKA, but at a vastly lower rate by p90^{RSK} (Fig. 1*A*) (13). p90^{RSK}, S6K1, MSK1, and PKA (all at ~1 unit/ml) phosphorylated GST-LKB1(KD) to 0.5–0.8 mol of phosphate/mol of protein after 60 min. Digestion of labeled GST-LKB1(KD) with trypsin, followed by chromatography on a C_{18} column, revealed that these kinases had phosphorylated GST-LKB1(KD) at one major tryptic phosphopeptide termed P1, eluting at 12.5% acetonitrile (Fig. 1*B*). Phosphoamino acid analysis revealed that peptide P1 contained only phosphoserine. After solid-phase sequencing, ^{32}P radioactivity was released after the third cycle of Edman degradation (data not shown). The molecular mass of P1 determined by MALDI-TOF mass spectrometry (862.400 Da) was identical to that expected for the tryptic phosphopeptide comprising residues 429–434 that is phosphorylated at Ser^{431} and in which Cys^{433} is pyridylethylated due to alkylation of LKB1 with 4-vinylpyridine prior to digestion with trypsin. This was confirmed by gas-phase Edman sequencing of this peptide (data not shown). Moreover, when Ser^{431} on GST-LKB1 was mutated to Ala, the resulting mutant was no longer phosphorylated significantly by p90^{RSK}, S6K1, MSK1, or PKA (Fig. 1*C*).

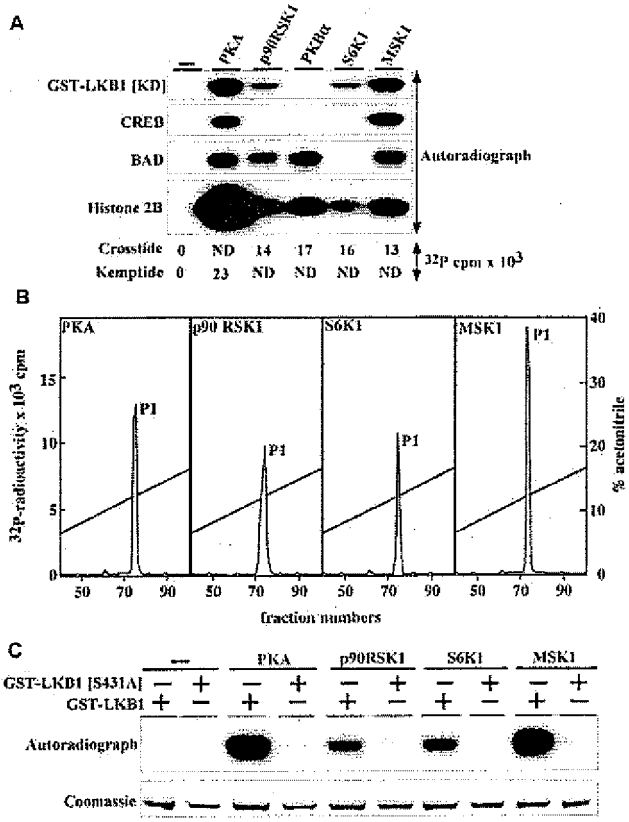

FIG. 1. **Phosphorylation of LKB1 at Ser^{431} by AGC kinase.** *A*, GST-LKB1(KD), GST-CREB, GST-BAD, or histone 2B and the peptide Crosstide or Kemptide were incubated with the indicated AGC kinase members in the presence of magnesium and [γ-^{32}P]ATP as described under "Experimental Procedures." Phosphorylation of protein substrates was determined following electrophoresis on a 4–12% gradient polyacrylamide gel, and the Coomassie Blue-stained bands corresponding to each substrate was autoradiographed. Phosphorylation of Crosstide and Kemptide was determined following adsorption of these peptides to phosphocellulose P-81 paper. *ND*, not determined. Similar results were obtained in three separate experiments. *B*, GST-LKB1(KD) that had been phosphorylated with the indicated kinases was digested with trypsin and chromatographed on a Vydac 218TP54 C_{18} column equilibrated in 0.1% (by volume) trifluoroacetic acid in water. The column was developed with a linear acetonitrile gradient (*diagonal lines*) at a flow rate of 0.8 ml/min, and fractions of 0.4 ml were collected. 80% of the radioactivity applied to the column was recovered from the major ^{32}P-containing peptide (peptide P1) at 12.5% acetonitrile. *C*, GST-LKB1 or GST-LKB1(S431A) expressed in *E. coli* was phosphorylated with the indicated AGC kinases as described for *A*. Similar results were obtained in two separate experiments.

Generation of a Phospho-specific Antibody That Recognizes LKB1 Phosphorylated at Ser^{431}—We prepared a phospho-specific antibody that recognized only LKB1 phosphorylated at Ser^{431}, termed antibody S431-P. Its specificity was established by the finding that it only recognized GST-LKB1 after phosphorylation *in vitro* by p90^{RSK1} and did not recognize GST-LKB1(S431A) (Fig. 2). Furthermore, the recognition of phosphorylated LKB1 was abolished when antibody S431-P was incubated with the phosphopeptide used to raise it, but not the non-phosphorylated form of this peptide (Fig. 2).

To identify cell lines that express significant levels of endogenous LKB1, we immunoblotted lysates derived from nine dif-

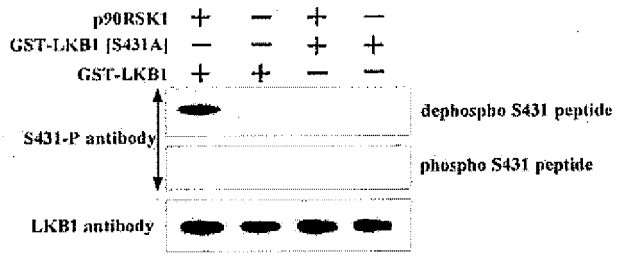

FIG. 2. **Generation of phospho-specific antibodies against LKB1.** Bacterially expressed GST-LKB1 or GST-LKB1(S431A) was incubated for 60 min with MgATP in the presence or absence of 1 unit/ml p90^{RSK1}. Aliquots containing 10 ng of GST-LKB1 were electrophoresed on a 4–12% gradient polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with antibody S431-P in the presence of either the phosphopeptide antigen used to raise this antibody (*phospho S431 peptide*) or the dephosphorylated form of this peptide (*dephospho S431 peptide*). The samples were also immunoblotted with the anti-LKB1 antibody raised against GST-LKB1. Similar results were obtained in at least three separate experiments.

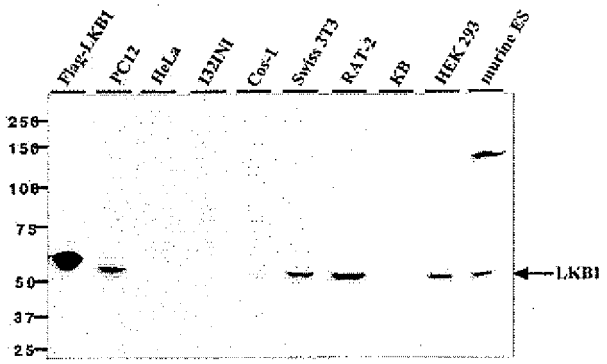

FIG. 3. **Expression of endogenous LKB1 in different cell lines.** Cell lysates from the indicated cell lines (20 μg) were electrophoresed on a 4–12% gradient polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with the anti-LKB1 antibody raised against the GST-LKB1 protein. As a control, a 293 cell lysate (0.3 μg) overexpressing FLAG epitope-tagged LKB1 was also immunoblotted. No LKB1 immunoreactive band was observed when the equivalent amount of 293 cell lysate not expressing FLAG-LKB1 was immunoblotted (data not shown). *PC12* refers to pheochromocytoma cells; *HEK 293* refers to human embryonic kidney cells; and *KB* refers to human oropharyngeal epidermoid carcinoma cells.

ferent cell lines with a polyclonal anti-LKB1 antibody raised against bacterially expressed GST-LKB1. This antibody recognized a single immunoreactive band migrating with slightly lower apparent molecular mass than FLAG epitope-tagged LKB1 (55 kDa) in most cell lines tested. These included NIH3T3 cells, which have previously been reported to express LKB1, but not HeLa cells, which have previously been reported not to express LKB1 (5, 10). We also failed to detect any expression of LKB1 in KB cells (Fig. 3). A similar pattern of expression of LKB1 was also observed using a different polyclonal anti-LKB1 antibody raised against an N-terminal region of LKB1 (data not shown). Rat-2 embryonic fibroblasts expressed the highest levels of LKB1 (Fig. 3) and were therefore used in the experiments described below.

Forskolin and a Cell-permeable Analog of cAMP Induce Phosphorylation of Endogenous LKB1 at Ser^{431}—Rat-2 cells were stimulated with the adenylate cyclase activator forskolin; the cells were lysed; and endogenous LKB1 was immunoprecipitated. The immunoprecipitates were immunoblotted with antibody S431-P as well as with an antibody recognizing the LKB1 protein to quantitate the amount of LKB1 immunoprecipitated. In unstimulated cells, the level of phosphorylation of LKB1 was low, but increased strikingly in response to forskolin, reaching a plateau within 2 min, which was maintained for 40 min (Fig. 4*A*). This phosphorylation is likely to be mediated by PKA, as the isoquinoline derivative H-89, which is a potent inhibitor of PKA (31, 32), largely prevented the forskolin-induced phosphorylation of LKB1 (Fig. 4*B*). Other signal transduction inhibitors, including three structurally unrelated inhibitors of MAPK kinase-1 activation (PD 98059 (33), PD 184352 (34), and U0126 (35)), an inhibitor of phosphatidylinositol 3-kinase (wortmannin (36)), and an inhibitor of S6K1 activation (rapamycin (37)), that would not be expected to affect PKA activation had no effect on phosphorylation of LKB1 induced by forskolin (Fig. 4*B*). Forskolin also induced phosphorylation of CREB at Ser^{133}, a known substrate of PKA, and this phosphorylation was also inhibited by H-89, but not by other signal transduction inhibitors (Fig. 4*B*). Stimulation of Rat-2 cells with the cell-permeable cAMP analog 8-(4-chlorophenylthio)-cAMP, which activates PKA, also induced phosphorylation of LKB1 at Ser^{431}, and this was inhibited by H-89 (Fig. 4*C*).

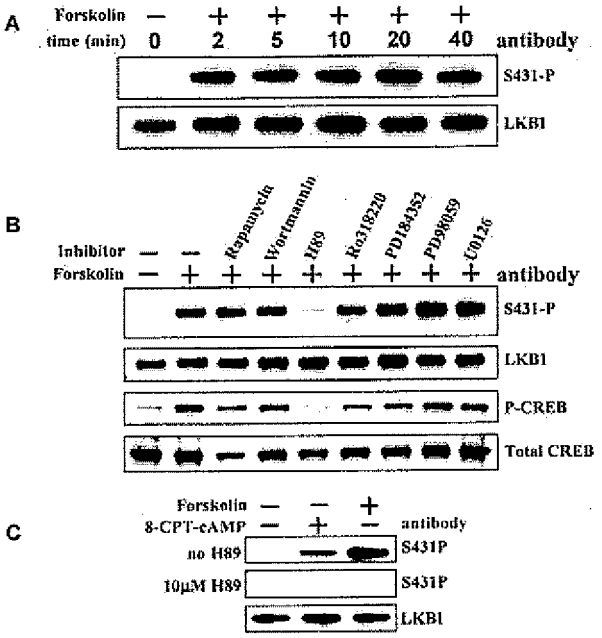

FIG. 4. **Phosphorylation of endogenous LKB1 at Ser^{431} is stimulated by forskolin.** *A*, Rat-2 cells were stimulated for the times indicated with 20 μM forskolin. The cells were lysed, and LKB1 was immunoprecipitated, subjected to electrophoresis on a 4–12% gradient polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with either antibody S431-P or the anti-LKB1 antibody raised against the GST-LKB1 protein. Similar results were obtained in three separate experiments. *B*, same as described for *A*, except that prior to stimulation with forskolin for 10 min, the cells were pretreated for 30 min with 0.1 μM rapamycin, 10 μM H-89, 5 μM Ro 318220, 2 μM PD 184352, 50 μM PD 98059, 1 μM U0126, or 0.1 μM wortmannin, except that this was added to the cells 10 min prior to stimulation. Cell lysates were also immunoblotted with a phospho-specific antibody that recognizes CREB phosphorylated at Ser^{133} (*P-CREB*) and with an antibody that recognizes the CREB protein (*Total CREB*). *C*, same as described for *A*, except that Rat-2 cells were stimulated for 10 min with 20 μM forskolin or 100 μM 8-(4-chlorophenylthio)-cAMP (*8-CPT-cAMP*) in the presence or absence of 10 μM H-89.

EGF Induces Phosphorylation of Endogenous LKB1 at Ser^{431}—EGF induced a substantial activation of p90^{RSK} (Fig. 5*A*), MSK1 (Fig. 5*B*), and S6K1 (Fig. 5*C*) in Rat-2 cells, as expected. The activation of p90^{RSK} and MSK1 was rapid and reached near-maximum levels within 5 min. However, whereas the activity of p90^{RSK} was only moderately reduced by 40 min, the activation of MSK1 was more transient and had decreased to near-basal levels by 40 min. As expected, the activation of p90^{RSK} and MSK1 was completely inhibited by incubating cells

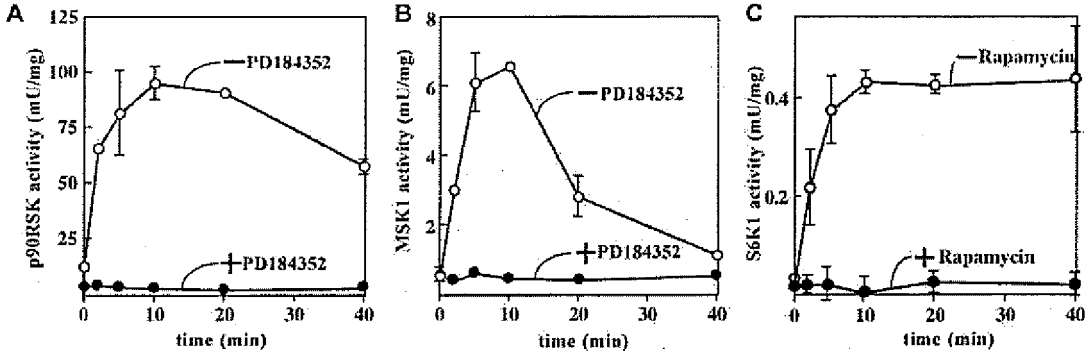

FIG. 5. **Activation of p90^{RSK}, MSK1, and S6K1 in EGF-stimulated Rat-2 cells.** Rat-2 cells were pretreated for 30 min in the presence (●) or absence (○) of 2 μM PD 184352 (*A* and *B*) or 100 nM rapamycin (*C*) prior to stimulation with 100 ng/ml EGF for the times indicated. The cells were lysed, and p90^{RSK} (*A*), MSK1 (*B*), and S6K1 (*C*) were immunoprecipitated from the same lysate and assayed. The data are presented as the means ± S.E. for two separate experiments, with each determination carried out in triplicate.

with PD 184352 prior to stimulation with EGF (Fig. 5, *A* and *B*). The activation of S6K1 by EGF was slower, reaching a plateau after 10 min. As expected, the activation of S6K1 was prevented by the immunosuppressant drug rapamycin (Fig. 5*C*) and the phosphatidylinositol 3-kinase inhibitor wortmannin (data not shown).

EGF stimulation of Rat-2 cells induced a significant phosphorylation of LKB1 at Ser^{431} within 2 min, which reached a maximum within 10 min before declining to lower levels by 40 min (Fig. 6*A*). PD 184352 (Fig. 6*A*) and PD 98059 and U0126 (Fig. 6*B*) completely inhibited EGF-induced phosphorylation of LKB1. In contrast, rapamycin and wortmannin had no effect on the EGF-mediated phosphorylation of LKB1 (Fig. 6*B*).

Ro 318220 is a bisindolylmaleimide that was originally developed as an inhibitor of protein kinase C, but that also inhibits p90^{RSK} (38) and MSK1 (39) with similar potency *in vitro*. In contrast, PKA is inhibited by Ro 318220 only at far higher concentrations (40, 41). Ro 318220 did not affect the forskolin-induced phosphorylation of LKB1 (Fig. 4*B*), but almost completely inhibited the EGF-stimulated phosphorylation of LKB1 (Fig. 6*B*).

Pharmacological Evidence That p90^{RSK} Mediates LKB1 Phosphorylation—The results presented above are consistent for a role for either p90^{RSK} or MSK1 in mediating the EGF-induced phosphorylation of LKB1 at Ser^{431}. Recent studies indicated that H-89 inhibits MSK1 (IC_{50} = 0.12 μM) with a similar potency to PKA (IC_{50} = 0.13 μM), but inhibition of p90^{RSK} is much weaker (IC_{50} = 2.6 μM) (40, 42). This indicates that cellular responses mediated by MSK1, but not those mediated by p90^{RSK}, should be sensitive to H-89 (42, 43). H-89 at a concentration of 5 μM had no detectable effect on the phosphorylation of LKB1 induced by EGF; and even at concentrations as high as 10 and 20 μM, H-89 had only a small effect (Fig. 7*A*). In contrast, the phosphorylation of CREB at Ser^{133} in response to EGF, which is thought to be mediated by MSK1 rather than p90^{RSK} (13, 24, 43), was virtually abolished even at 5 μM H-89 (Fig. 7*A*). Consistent with previous studies in other cells, the EGF-induced phosphorylation of CREB in Rat-2 cells was inhibited by PD 184352 or Ro 318220 (Fig. 6*B*). Similarly, 20 μM H-89 had no effect on the activation of p90^{RSK} (Fig. 7*B*) and MSK1 (Fig. 7*C*) induced by EGF after these kinases were immunoprecipitated from cells and assayed in the absence of H-89 (Fig. 7*B*).

Genetic Evidence That p90^{RSK} Rather than MSK1 Mediates LKB1 Phosphorylation in Vivo—p90^{RSK}, in addition to requiring phosphorylation by ERK1/2, also needs to be phosphorylated at Ser^{222} (a site phosphorylated by PDK1) (14, 45) to become activated (44). We have recently prepared mouse ES cells deficient in the expression of PDK1 (termed PDK1$^{-/-}$ cells); and as expected, these cells possessed no detectable p90^{RSK} activity even after TPA stimulation, which activates ERK1/2 in these cells (25). Importantly, in PDK1$^{-/-}$ ES cells, TPA still induced activation of MSK1 to the same extent as observed in control ES cells (25). Recently, MSK1-deficient ES cells have also been generated; and in these cells, TPA failed to stimulate the phosphorylation of CREB, despite p90^{RSK} being activated normally (24).

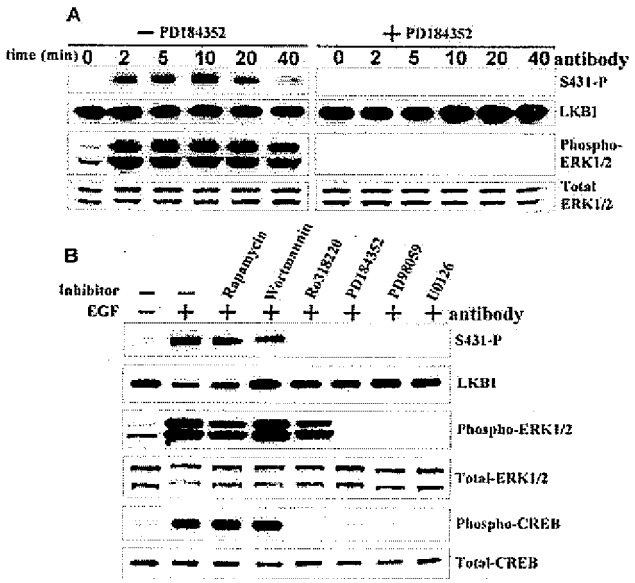

FIG. 6. **Effect of signal transduction inhibitors on phosphorylation of LKB1 induced by EGF.** *A*, Rat-2 cells were pretreated for 30 min in the presence or absence of 2 μM PD 184352 prior to stimulation with 100 ng/ml EGF for the times indicated. The cells were lysed, and LKB1 was immunoprecipitated, subjected to electrophoresis on a 4–12% gradient polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with either antibody S431-P or the anti-LKB1 antibody raised against the GST-LKB1 protein. Cell lysates (20 μg of protein) from these stimulations were also immunoblotted with a phospho-specific antibody that recognizes the activated forms of ERK1 and ERK2 (*Phospho-ERK1/2*) as well as with an antibody that recognizes ERK1 and ERK2 proteins (*Total ERK1/2*). Similar results were obtained in three separate experiments. *B*, same as described for *A*, except prior to stimulation of Rat-2 cells with 100 ng/ml EGF for 10 min, the cells were pretreated for 30 min with 0.1 μM rapamycin, 5 μM Ro 318220, 2 μM PD 184352, 50 μM PD 98059, 1 μM U0126, or 0.1 μM wortmannin, except that this was added to the cells 10 min prior to stimulation. Cell lysates were also immunoblotted with a phospho-specific antibody that recognizes CREB phosphorylated at Ser^{133} (*Phospho-CREB*) and with an antibody that recognizes the CREB protein (*Total-CREB*).

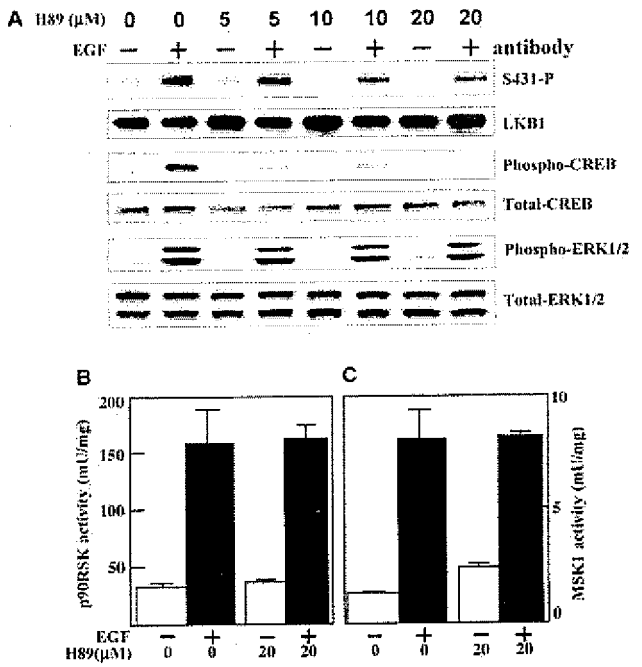

FIG. 7. **Effect of H-89 on phosphorylation of LKB1 and CREB induced by EGF.** Prior to stimulation of Rat-2 cells with 100 ng/ml EGF for 10 min, the cells were pretreated for 30 min with the indicated concentrations of H-89. *A*, LKB1 was immunoprecipitated, subjected to electrophoresis on a 4–12% gradient polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with either antibody S431-P or the anti-LKB1 antibody raised against the GST-LKB1 protein. Cell lysates (20 μg of protein) from these stimulations were also immunoblotted with a phospho-specific antibody that recognizes CREB phosphorylated at Ser^{133} (*Phospho-CREB*) and with an antibody that recognizes the CREB protein (*Total-CREB*). The lysates were also immunoblotted with an antibody that recognizes the activated forms of ERK1 and ERK2 (*Phospho-ERK1/2*) as well as with an antibody that recognizes ERK1 and ERK2 proteins (*Total-ERK1/2*). Similar results were obtained in three separate experiments. *B* and *C*, p90^{RSK} and MSK1, respectively, were immunoprecipitated and assayed. The data are presented as the means ± S.E. for two separate experiments, with each determination carried out in triplicate.

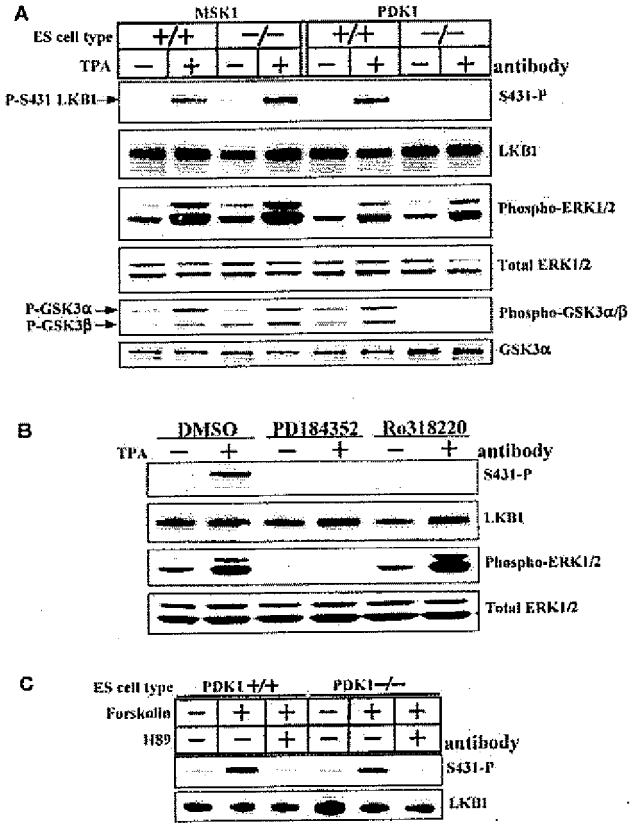

FIG. 8. **Phosphorylation of endogenous LKB1 in MSK1$^{-/-}$ and PDK1$^{-/-}$ ES cells.** *A*, the indicated ES cell lines were stimulated for 20 min with 400 ng/ml TPA. The cells were lysed, and LKB1 was immunoprecipitated, subjected to electrophoresis on a 4–12% gradient polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with either antibody S431-P or the anti-LKB1 antibody raised against the GST-LKB1 protein. The lysates (20 μg of protein) were also immunoblotted with an antibody that recognizes the activated forms of ERK1 and ERK2 (*Phospho-ERK1/2*) as well as with an antibody that recognizes ERK1 and ERK2 proteins (*Total ERK1/2*). The lysates were also immunoblotted with an antibody that recognizes GSK3α phosphorylated at Ser^{21} and GSK3β phosphorylated at Ser^{9} (*Phospho-GSK3α/β*) as well as with an antibody that recognizes GSK3α (*GSK3α*). *B*, same as described for *A*, except that the cells were incubated in the presence or absence of PD 184352 (2 μM), or Ro 318220 (5 μM), or dimethyl sulfoxide (DMSO) as a control for 30 min prior to stimulation with TPA. *C*, same as described for *A*, except that the cells were incubated in the presence or absence of H-89 (10 μM) and then stimulated with 20 μM forskolin for 10 min.

We therefore decided to investigate whether TPA induced the phosphorylation of LKB1 in PDK1$^{-/-}$ and MSK1$^{-/-}$ ES cells. LKB1 was expressed in control mouse ES cells (Fig. 3), and we demonstrate in Fig. 8*A* that TPA induced the phosphorylation of LKB1 at Ser^{431} in both the control and MSK1$^{-/-}$ ES cell lines, but not in the PDK1$^{-/-}$ ES cell line. The phosphorylation of LKB1 in these cells, like that observed in response to EGF in Rat-2 cells, was inhibited by either PD 184352 or Ro 318220 (Fig. 8*B*). ERK1 and ERK2 were activated by TPA in both the PDK1$^{-/-}$ and MSK1$^{-/-}$ ES cells (Fig. 8*A*) in a PD 184352-sensitive, but Ro 318220- and H-89-insensitive manner (Fig. 8*B*). p90^{RSK} is thought to mediate the TPA-stimulated phosphorylation of GSK3α at Ser^{21} and of GSK3β at Ser^{9} (46, 47). We demonstrate in Fig. 8*A* that the phosphorylation of GSK3α and GSK3β was stimulated by TPA in MSK1$^{-/-}$ ES cells. In contrast, no detectable phosphorylation of GSK3α or GSK3β was observed in either unstimulated or TPA-stimulated PDK1$^{-/-}$ ES cells (Fig. 8*A*), as reported previously (25). Stimulation of the control, PDK1$^{-/-}$, and MSK1$^{-/-}$ ES cell lines with forskolin induced a potent phosphorylation of LKB1 at Ser^{431}. Consistent with this being mediated by PKA, it was inhibited by H-89, but not by PD 184352 or Ro 318220 (Fig. 8*C*).

Evidence That Phosphorylation of LKB1 at Ser^{431} Does Not Affect Its Activity—No substrates for LKB1 have been identified thus far, and the only assay that has been used to gauge LKB1 activity has been to measure its autophosphorylation. We have confirmed that recombinant wild-type GST-LKB1, but not GST-LKB1(KD), expressed in 293 cells autophosphorylated in the presence of MnATP (Fig. 9*A*), but not MgATP (data not shown), as reported by others (5, 48). We also demonstrated that the extent of autophosphorylation of wild-type GST-LKB1 was comparable to that of GST-LKB1(S431D) and GST-LKB1(S431A). We have also tested 25 peptides and 50 proteins routinely used to assay protein kinases and found just one, *viz.* the p53 tumor suppressor protein, that was phosphorylated *in vitro* by wild-type LKB1, but not by a catalytically inactive mutant (Fig. 9*A*). The extent to which p53 was phosphorylated by wild-type GST-LKB1 was similar to that to which it was phosphorylated by GST-LKB1(S431D) and GST-LKB1(S431A) (Fig. 9*A*). As many protein kinases phosphorylate p53 *in vitro*, but not *in vivo*, further work is required to establish whether p53 is a physiological substrate for LKB1. However, this finding was useful for the development of an assay for LKB1 activity. In Fig. 9*B*, we demonstrate that stimulation of Rat-2

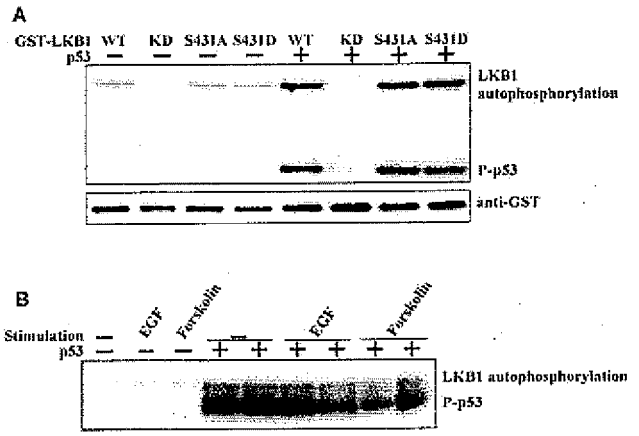

FIG. 9. **Evidence that phosphorylation of LKB1 at Ser^{431} does not affect its activity.** *A*, wild-type (*WT*) GST-LKB1 or the indicated mutants of GST-LKB1 expressed in 293 cells were incubated for 30 min with manganese/[γ-^{32}P]ATP in the presence or absence of mouse p53 (2 μg) and electrophoresed on a 4–12% gradient polyacrylamide gel, which was autoradiographed. The samples were also immunoblotted with antibodies recognizing the GST tag to ensure that comparable amounts of wild-type and mutant GST-LKB1 were used. *B*, Rat-2 cells were stimulated for 10 min with 100 ng/ml EGF or 20 μM forskolin or were left unstimulated. The cells were lysed, and LKB1 was immunoprecipitated and assayed by incubation for 30 min with manganese/[γ-^{32}P]ATP in the presence or absence of p53 to measure autophosphorylation activity.

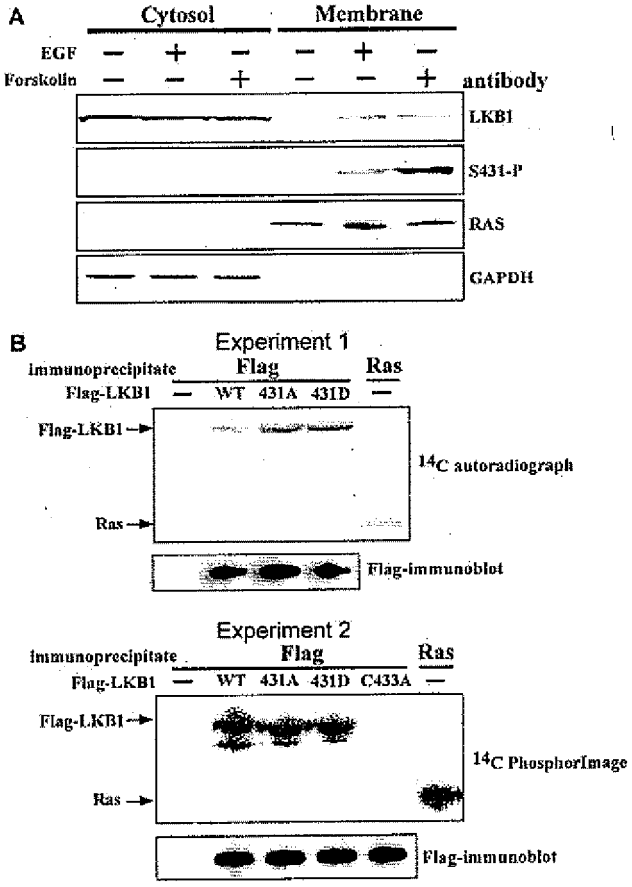

FIG. 10. **Evidence that phosphorylation of Ser^{431} does not affect membrane association or prenylation of LKB1.** *A*, Rat-2 cells were left unstimulated or stimulated for 30 min with 100 ng/ml EGF or 20 μM forskolin. The cells were lysed in a lysis buffer without Triton X-100 (Buffer D), and cytosolic and membrane fractions were prepared as described under "Experimental Procedures." The membrane fraction was washed with Buffer D containing 0.5 M NaCl and then resuspended in lysis buffer containing Triton X-100 (Buffer C). The cytosol and membrane were immunoblotted with the anti-LKB1 antibody raised against the GST-LKB1 protein (40 μg of protein), antibody S431-P (20 μg of protein), an antibody that recognizes all Ras isoforms (10 μg of protein), or an antibody that recognizes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; 5 μg of protein). Similar results were obtained in two separate experiments. *B*, 293 cells were transfected with wild-type (*WT*) FLAG-LKB1, the indicated mutant forms of FLAG-LKB1, or empty pCMV5 vector. The cells were labeled with [^{14}C]mevalonic acid for 24 h and lysed in Buffer C, and LKB1 and Ras were immunoprecipitated with the anti-FLAG or anti-Ras antibody. 90% of the immunoprecipitate was electrophoresed on 4–12% polyacrylamide gel, transferred to nitrocellulose, and autoradiographed for ^{14}C radioactivity. The remaining 10% of the immunoprecipitate was immunoblotted with the anti-FLAG antibody to monitor the amount of wild-type and mutant FLAG-LKB1 in each immunoprecipitate. The results of two separate experiments are shown.

cells with forskolin and EGF did not affect the extent to which the endogenous LKB1 immunoprecipitated from these cells autophosphorylated or the degree to which it phosphorylated p53.

Evidence That a Small Pool of Endogenous LKB1 Associates with Membranes—Although one previous study has indicated that the C-terminal fragment of LKB1, when transfected into cells, is prenylated and localized at cell membranes (10), other localization studies of full-length LKB1 expressed in various cell lines have indicated that LKB1 is expressed in both the nucleus and cytoplasm rather than at the plasma membrane (5, 7). To investigate whether endogenously expressed LKB1 is associated with cell membranes, we prepared cytosolic and membrane fractions of unstimulated Rat-2 cells or Rat-2 cells stimulated with EGF or forskolin. An equal amount of cytosolic protein and membrane protein was immunoblotted with antibodies recognizing LKB1, LKB1 phosphorylated at Ser^{431}, Ras (a prenylated membrane protein), and glyceraldehyde-3-phosphate dehydrogenase (a cytosolic protein). As expected, Ras was localized exclusively in the membrane fraction, whereas glyceraldehyde-3-phosphate dehydrogenase was localized only in the cytoplasmic fraction. Although LKB1 was mainly localized in the cytosol, there was a small but significant amount of LKB1 associated with the membrane fraction (Fig. 10). Stimulation of Rat-2 cells with EGF and forskolin did not significantly alter the amount of LKB1 localized at the membrane; but interestingly, phosphorylation of LKB1 at Ser^{431} was detected only in the membrane fraction, and not in the cytosolic fraction (Fig. 10*A*).

Evidence That Phosphorylation of Ser^{431} Does Not Affect Prenylation of LKB1—To establish whether full-length LKB1 expressed in cells was prenylated, we transfected 293 cells with wild-type FLAG-LKB1. The cells were metabolically labeled with [^{14}C]mevalonic acid (a precursor in isoprenoid biosynthesis) for 24 h and lysed. FLAG-LKB1 was immunoprecipitated with an anti-FLAG antibody, and Ras was also immunoprecipitated as a control. Wild-type FLAG-LKB1 and Ras were significantly ^{14}C-labeled, indicating that they were prenylated (Fig. 10*B*). FLAG-LKB1(S431A) and FLAG-LKB1(S431D) expressed in 293 cells were ^{14}C-labeled to the same degree as wild-type LKB1, suggesting that phosphorylation of LKB1 at Ser^{431} does not affect prenylation of LKB1. A mutant of LKB1 in which the conserved Cys residue predicted to be a prenyl acceptor residue of LKB1 was mutated to Ala (FLAG-LKB1(C433A)) was not ^{14}C-labeled (Fig. 10*B*), confirming that Cys^{433} is likely to be the site of prenylation.

Evidence That Phosphorylation of LKB1 at Ser^{431} Is Required for Its Ability to Inhibit Cell Growth—Makela and coworkers (5) have demonstrated that expression of wild-type LKB1, but not of a catalytically inactive mutant of LKB1, in G361 melanoma cells, which do not express LKB1, potently

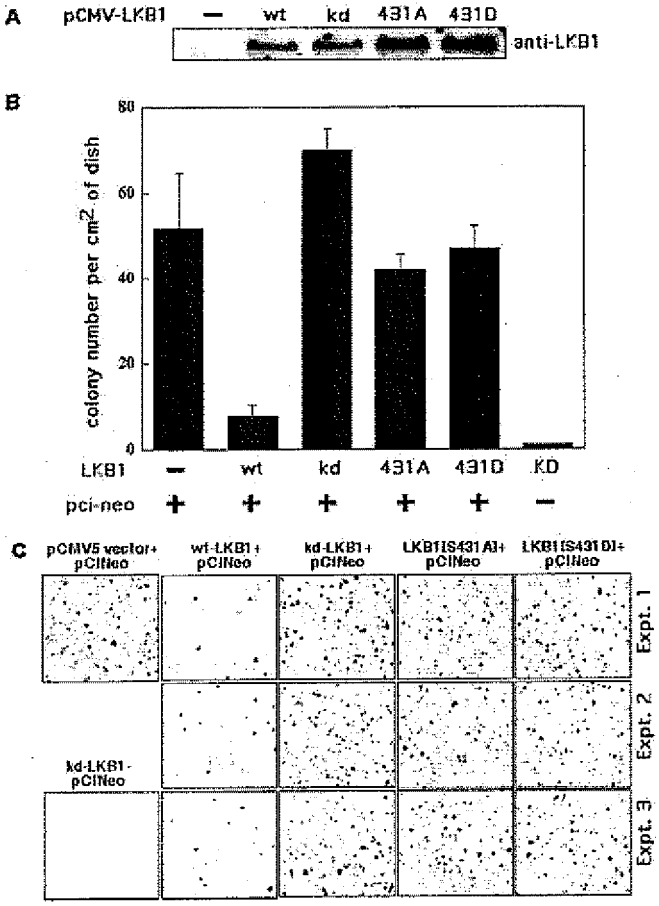

FIG. 11. **Evidence that phosphorylation of LKB1 at Ser^{431} is necessary for its ability to inhibit cell growth.** G361 cells were transfected with the indicated wild-type (*wt*) and mutant forms of FLAG-LKB1 in the presence or absence of the pCI-neo expression vector, which encodes for G418 resistance. After 4 days, the samples were immunoblotted with the anti-LKB1 antibody to ensure that comparable amounts of wild-type and mutant forms of LKB1 were expressed (*A*). After 16 days of G418 selection, Giemsa-stained colonies were counted (*B*) and photographed (*C*). The S.D. values and photographs are from three independent dishes. Similar results were obtained in two separate experiments, with each condition carried out in triplicate.

suppresses the ability of these cells to grow. We have confirmed that G361 cells do not express LKB1 (Fig. 11*A*). To determine whether mutation of Ser^{431} of LKB1 to either Ala or Asp affected the ability of LKB1 to suppress growth of G361 cells, we transfected these cells with an expression vector encoding either wild-type LKB1 or catalytically inactive LKB1 (LKB1(S431A) or LKB1(S431D)) together with a plasmid encoding a neomycin/G418 resistance gene using the same protocol as Makela and co-workers (5). After 16 days of selection with G418, as expected from the previous study (5), a 10-fold lower number of colonies were recovered when the G361 cells were transfected with a plasmid encoding wild-type LKB1 compared with catalytically inactive LKB1 (Fig. 11). However, when the G361 cells were transfected with LKB1(S431A) or LKB1(S431D), a 7-fold greater number of colonies were obtained compared with transfections with wild-type LKB1 (Fig. 11). This indicates that phosphorylation of LKB1 at Ser^{431} is likely to play a role in enabling LKB1 to inhibit cell growth.

Role of Prenylation in Regulating LKB1 Function—To investigate whether prenylation of LKB1 was required for its ability to suppress cell growth, we compared the ability of wild-type LKB1 and the mutant of LKB1 that cannot be prenylated (LKB1(C433A)) to prevent the growth of G361 cells. In Fig. 12 (*A* and *B*), we demonstrate that LKB1(C433A) was equally efficient at suppressing growth of G361 cells as wild-type LKB1, indicating that the prenylation of LKB1 is not essential for it to suppress the growth of these cells. To establish whether prenylation was required for LKB1 to be phosphorylated at Ser^{431}, we transfected 293 cells with wild-type and mutant LKB1. Stimulation of these cells with either EGF to activate p90^{RSK} or forskolin to activate PKA induced significant phosphorylation of wild-type LKB1 and the non-prenylated mutant, LKB1(C433A) (Fig. 12*B*). As controls, we show that EGF and forskolin induced phosphorylation of LKB1(KD), but not of LKB1(S431A) (Fig. 12*B*). Purified GST-LKB1(C433A) phosphorylated itself and p53 *in vitro* to the same extent as wild-type GST-LKB1 (Fig. 12*C*), indicating that prenylation of LKB1 is not required for the activity of the enzyme *in vitro*.

Cys^{433} Is Modified by Farnesylation—We decided to isolate the LKB1 tryptic peptide containing Cys^{433} and to determine its mass to establish whether it was modified by farnesylation or geranylgeranylation. As the tryptic peptide containing Cys^{433} will be in the same peptide as Ser^{431}, we decided to ^{32}P label 293 cells expressing wild-type LKB1, LKB1(S431A), and LKB1(C433A); stimulate them with forskolin; immunoprecipitate LKB1; and perform standard tryptic peptide map analysis to purify the tryptic peptide containing Ser^{431} and Cys^{433}. These experiments demonstrated that forskolin stimulated phosphorylation of both wild-type LKB1 and mutant LKB1(C433A), but not mutant LKB1(S431A) (Fig. 13*A*). ^{32}P-Labeled LKB1 from these experiments was digested with trypsin, and the resulting peptides were separated by chromatography on a C_{18} column. A number of minor ^{32}P-labeled peptides were recovered from wild-type and mutant LKB1 derived from unstimulated cells (Fig. 13, *B–D*). Forskolin stimulated the phosphorylation of two peptides of wild-type LKB1; one termed peptide P_A, eluting at 12.5% acetonitrile (the same position as peptide P1 in Fig. 1*B*), and the other termed peptide P_B, eluting at 48% acetonitrile (Fig. 13*B*). Peptide P_A corresponds to the tryptic phosphopeptide comprising residues 429–434 that is phosphorylated at Ser^{431} and in which Cys^{433} is pyridylethylated because of the alkylation of free Cys residues of LKB1 with 4-vinylpyridine prior to digestion with trypsin. The mass of this peptide determined by MALDI-TOF mass spectrometry is 862.400 (the predicted mass for this peptide is 862.401), and it contains phosphoserine. ^{32}P radioactivity was released at the third cycle of solid-phase Edman sequencing (data not shown). As expected, peptide P_A was absent from the tryptic peptide map derived from forskolin-stimulated LKB1(S431A), whereas this peptide eluted slightly earlier on the C_{18} column from the forskolin-stimulated LKB1(C433A) sample, as it was not pyridylethylated (the mass of this peptide was determined as 725.3721 Da, coinciding with the predicted mass of 725.3711 Da for the tryptic phosphopeptide comprising residues 429–434 that is phosphorylated at Ser^{431} and in which the residue equivalent to Cys^{433} is an Ala). Peptide P_B was observed in the tryptic peptide map derived from forskolin-stimulated wild-type LKB1, but was not observed in the maps from forskolin-stimulated LKB1(S431A) and LKB1(C433A) (Fig. 13, *B–D*). The mass of peptide P_B is 961.5319 Da (Fig. 13*E*), identical to that of the peptide comprising residues 429–434 of LKB1 (Arg-Leu-Ser-Ala-Cys-Lys) that is phosphorylated at Ser^{431} and farnesylated at Cys^{433} (predicted mass of 961.5310 Da). Had this peptide been geranylgeranylated, its mass would have been 68.070 Da higher. Consistent with this analysis, peptide P_B contains phosphoserine, and solid-phase sequencing indicated that ^{32}P radioactivity was released after the third cycle of Edman sequencing (data not shown). This also confirms the

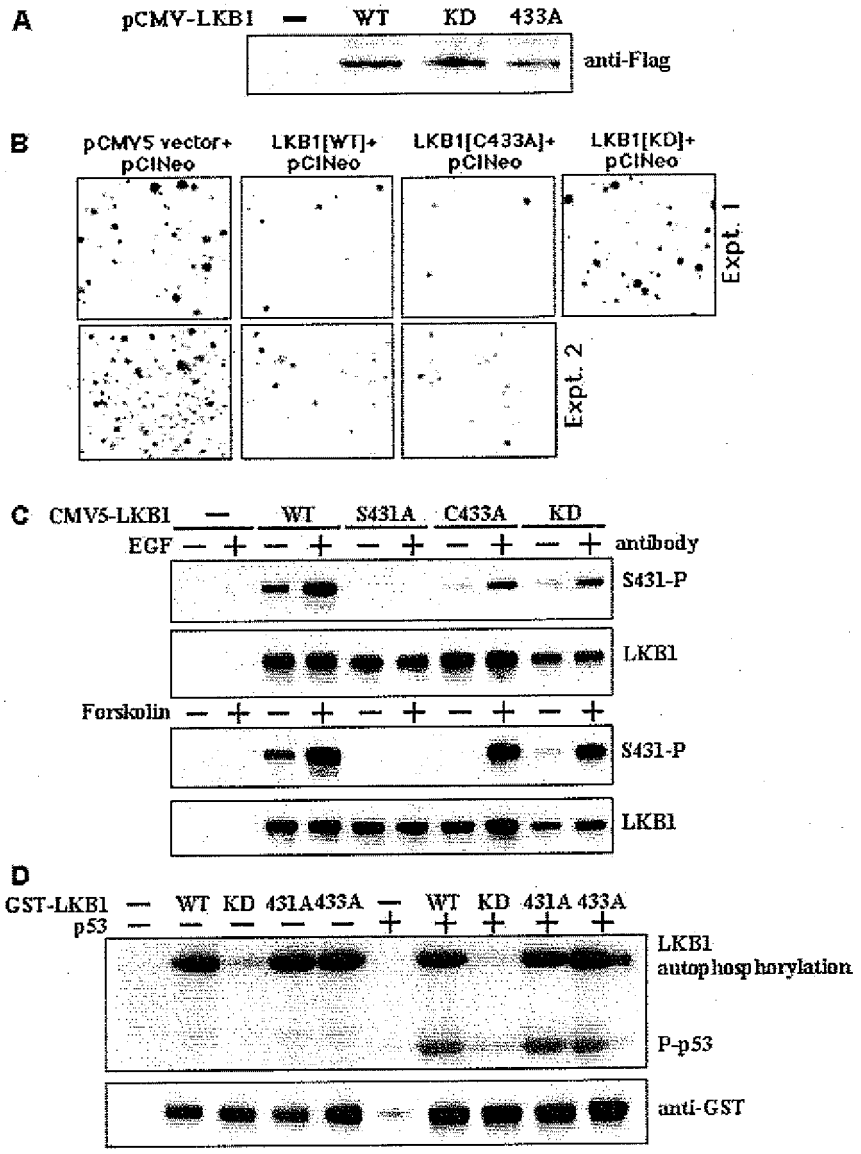

FIG. 12. **Role of prenylation of LKB1 in regulating phosphorylation of Ser^{431} and activity and ability of LKB1 to suppress cell growth.** G361 cells were transfected with the indicated wild-type (*WT*) and mutant forms of FLAG-LKB1 in the presence or absence of the pCI-neo expression vector, which encodes for G418 resistance. *A*, after 4 days, the samples were immunoblotted with the anti-LKB1 antibody to ensure that comparable amounts of wild-type and mutant forms of GST-LKB1 were expressed. *B*, after 16 days of G418 selection, Giemsa-stained colonies were photographed. *C*, 293 cells were transfected with the indicated N-terminal FLAG epitope-tagged wild-type and mutant forms of LKB1 or the empty pCMV5 vector (−) as a control. After 24 h, the cells were deprived of serum overnight and left unstimulated or were stimulated for 10 min with 100 ng/ml EGF or 20 μM forskolin. The cells were lysed, and 1 μg of cell lysate was immunoblotted with antibody S431-P to measure phosphorylation of LKB1 at Ser^{431} or with the anti-FLAG antibody to assess the level of expression of LKB1 in the lysate. *D*, wild-type GST-LKB1 or the indicated mutants of GST-LKB1 expressed in 293 cells were incubated for 30 min with manganese/[γ-^{32}P]ATP in the presence or absence of mouse p53 (2 μg) and electrophoresed on a 4–12% gradient polyacrylamide gel, which was autoradiographed. The samples were also immunoblotted with antibodies recognizing the GST tag to ensure that comparable amounts of wild-type and mutant forms of GST-LKB1 were used. Similar results were obtained in two separate experiments for all data presented, with each condition in *B* carried out in triplicate.

mass spectrometry analysis result that peptide P_B is not carboxymethylated; otherwise, it would not be possible to couple it through its C-terminal carboxyl residue to the arylamine residue for solid-phase sequencing, and it would have an observed mass of 16.0 Da greater. In the fraction adjacent to peptide P_B in forskolin-stimulated wild-type LKB1, the non-Ser^{431}-phosphorylated peptide comprising residues 429–434 of LKB1 that is farnesylated at Cys^{433} with a mass of 881.576 Da (predicted mass of 881.5647 Da) was also observed (Fig. 13*F*).

DISCUSSION

One of the major findings of this study is that we identified Ser^{431} as an *in vivo* phosphorylation site in LKB1. In support of a role of PKA in mediating the phosphorylation of LKB1, we found that stimulation of Rat-2 cells (Fig. 4) and embryonic stem cells (Fig. 8*B*) with forskolin, an activator of PKA, induced the phosphorylation of endogenous LKB1 at Ser^{431} and that this was inhibited by H-89 (Fig. 8). EGF also induced the phosphorylation of Ser^{431}, and this is likely to be mediated by p90^{RSK}. This event is prevented by inhibitors of MAPK kinase-1 activation and an inhibitor of p90^{RSK} and MSK1 (Ro 318220), but not by concentrations of H-89 that selectively inhibit MSK1. We supported this finding by demonstrating that, in control ES cell lines and in an MSK1-deficient ES cell line (24), TPA potently activated p90^{RSK} in these cells and still induced the phosphorylation of LKB1 at Ser^{431} and that this phosphorylation was inhibited by PD 184352 and Ro 318220, but not by H-89 (Fig. 8). In contrast, in a PDK1$^{-/-}$ ES cell line (25), in which TPA activated ERK1/2 and MSK1, but not p90^{RSK}, this agonist failed to induce phosphorylation of LKB1 at Ser^{431} (Fig. 8). Therefore, the combined pharmacological and genetic data that we have obtained in Rat-2 and ES cells, which are summarized in Fig. 14, strongly support a role for p90^{RSK}, rather than MSK1 or S6K1, in mediating the phosphorylation of LKB1 at Ser^{431} in response to agonists that activate ERK1/2.

Interestingly, MSK1 phosphorylated LKB1 *in vitro* at an ~2-fold higher initial rate than p90^{RSK} or S6K1; but under identical conditions, MSK1 phosphorylated CREB at an ~100-fold higher rate than p90^{RSK} (Fig. 1*A*). Previous work has shown that there is typically a 20-fold higher level of p90^{RSK} activity in cells compared with MSK1 activity (13, 43). This is also the case in Rat-2 cells, in which, after 5 min of EGF stimulation, MSK1 activity reached ~6 milliunits/mg (Fig. 5*A*) and p90^{RSK} activity reached ~100 milliunits/mg (Fig. 5*B*). The larger amount of p90^{RSK} activity in cells may explain why

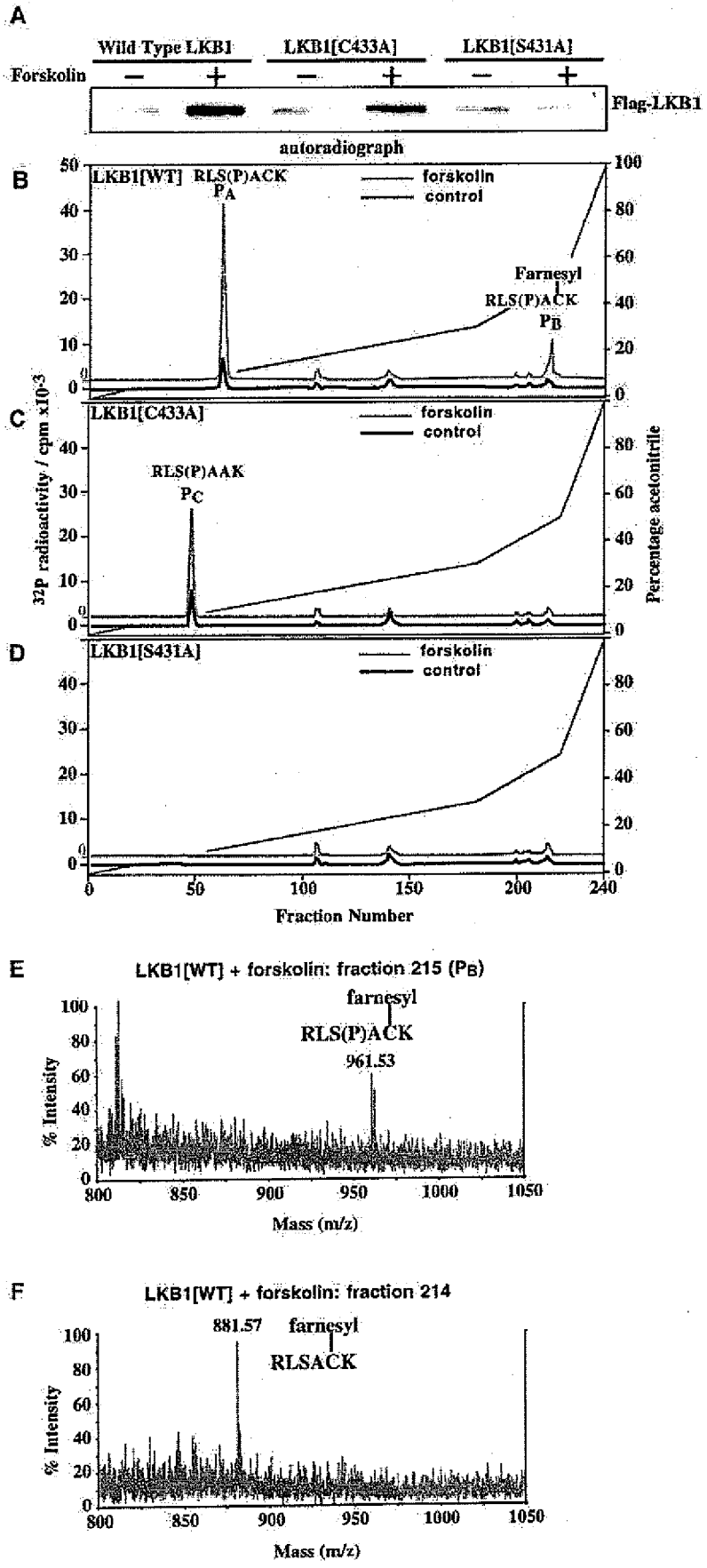

FIG. 13. **LKB1 is farnesylated at Cys^{433}.** 293 cells were transiently transfected with DNA constructs encoding wild-type (*WT*) FLAG-LKB1 or mutant LKB1(S431A) or LKB1(C433A). After 24 h, the cells were deprived of serum overnight and then washed in phosphate-free medium and incubated for 3 h with ^{32}P-labeled inorganic phosphate. The cells were either left unstimulated or stimulated for 10 min with 20 μM forskolin. The cells were lysed; the ^{32}P-labeled LKB1 protein was immunoprecipitated from the lysates using an anti-FLAG antibody, treated with 4-vinylpyridine, and electrophoresed on a 4–12% polyacrylamide gel; and ^{32}P-labeled LKB1 was visualized by autoradiography (*A*). ^{32}P-Labeled LKB1 was excised from the gel, digested with trypsin, and chromatographed on a Vydac 218TP54 C_{18} column equilibrated in 0.1% (by volume) trifluoroacetic acid, and the columns were developed with a linear acetonitrile gradient (*diagonal lines*). The flow rate was 0.8 ml/min, and fractions of 0.4 ml were collected (*B–D*). The two major forskolin-stimulated ^{32}P-labeled peptides eluting at 12.5% (peptide P_A) and 48% (peptide P_B) acetonitrile are indicated. The MALDI-TOF mass spectrum profile of fraction 215, in which peptide P_B derived from forskolin-stimulated wild-type LKB1, corresponds to residues 429–434 of LKB1 (Arg-Leu-Ser-Ala-Cys-Lys) that is phosphorylated at Ser^{431} and farnesylated at Cys^{433} (*E*). The MALDI-TOF mass spectrum profile of fraction 214 is also shown, which contains the peptide comprising residues 429–434 of LKB1 that is farnesylated at Cys^{433}, but that is not phosphorylated at Ser^{431} (*F*).

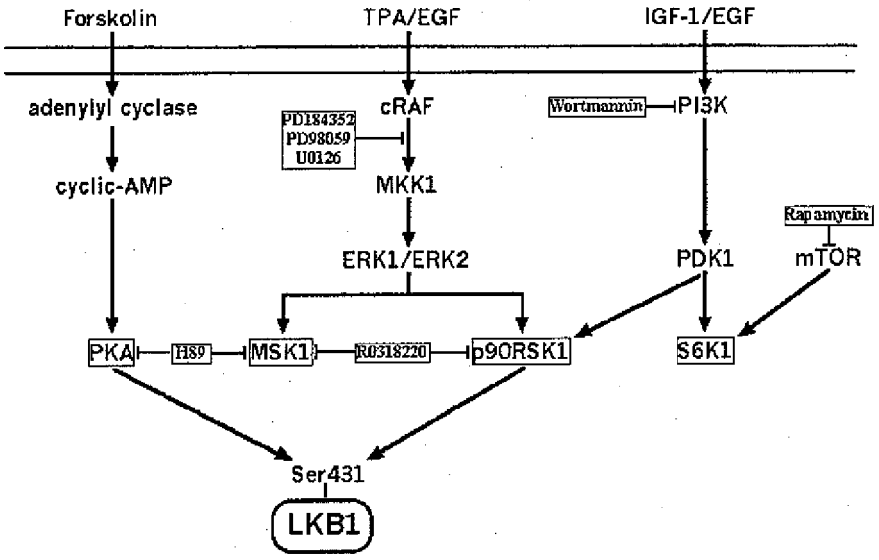

FIG. 14. **LKB1 is phosphorylated at Ser^{431} in response to agonists that activate p90^{RSK} and PKA.** The signal transduction pathways and the sites of action of the kinase inhibitors used in this study are indicated. *IGF-1*, insulin-like growth factor-1; *PI3K*, phosphatidylinositol 3-kinase.

p90^{RSK} rather than MSK1 phosphorylated LKB1 *in vivo*. Thus, in general, substrates that are phosphorylated *in vitro* at a similar initial rate by MSK1 and p90^{RSK} are perhaps more likely to be physiological substrates for p90^{RSK} rather than for MSK1. In contrast, it could be expected that *in vivo* MSK1 substrates such as CREB will turn out to be vastly superior *in vitro* substrates for MSK1 compared with p90^{RSK}.

To determine whether the activation of MSK1 in the absence of p90^{RSK} activity could induce phosphorylation of LKB1, we stimulated Rat-2 cells with cellular stresses, including UV irradiation, hydrogen peroxide, and sorbitol, which activate MSK1 to the same extent as EGF, but do not activate p90^{RSK}. None of these agonists induced a significant phosphorylation of LKB1 at Ser^{431} (data not shown), further indicating that MSK1 does not phosphorylate LKB1 *in vivo*. It should also be noted that stimulation of Rat-2 cells with insulin-like growth factor-1, which potently activates S6K1, but not p90^{RSK} and MSK1, also did not induce a notable phosphorylation of LKB1 at Ser^{431} (data not shown). This is consistent with the observation that S6K1, in the absence of p90^{RSK} and MSK1 activity, does not phosphorylate LKB1 *in vivo* (data not shown). Ser^{431} does not lie in a consensus motif required for phosphorylation by PKB (Arg-Xaa-Arg-Xaa-Xaa-(Ser/Thr)) (11) and would not be expected to be phosphorylated by PKB. However, another residue on LKB1 (Thr^{336}) lies in a consensus motif for phosphorylation by PKB, and this site is conserved in the *Xenopus* LKB1 homolog XEEK1. The finding that bacterially expressed GST-LKB1 (Fig. 1*A*) or GST-LKB1(KD) prepared from serum-starved transfected 293 cells, in which PKB is inactive (data not shown), was not significantly phosphorylated by PKB *in vitro* suggests that Thr^{336} may not be a physiological PKB phosphorylation site.

Previous studies have indicated that LKB1 may have a very narrow substrate specificity, as it did not phosphorylate a number of substrates routinely used to assay protein kinases (3–5). An alternative explanation is that LKB1 requires a regulatory subunit for activity; and thus, the catalytic subunit, when expressed alone, may possess only a basal level of activity. We found that only one substrate out of nearly 80 that we tested, *viz.* the p53 tumor suppressor protein, was phosphorylated by LKB1 prepared from transfected 293 cells, albeit at a low rate. A catalytically inactive mutant of GST-LKB1 in which a single residue had been mutated was unable to phosphorylate p53 (Fig. 9*B*). p53 was also phosphorylated *in vitro* by endogenous LKB1 immunoprecipitated from Rat-2 cells. Furthermore, phosphorylation of p53 occurred only in the presence of MnATP, but not MgATP, consistent with other studies showing that LKB1 is only active in the presence of MnATP. These observations suggest that LKB1, rather than a contaminating kinase, was the enzyme phosphorylating p53 in these experiments. GST-LKB1 expressed in *E. coli*, unlike GST-LKB1 expressed in 293 cells, did not phosphorylate itself or p53 (data not shown), indicating either that the bacterially expressed enzyme is misfolded or that LKB1 expressed in mammalian cells undergoes some modification or interaction with a regulatory component that activates it. Clearly, further studies are required to establish whether p53 is a physiological substrate for LKB1.

The finding that LKB1 autophosphorylation or its activity for p53 was not altered following mutation of Ser^{431} to Asp or Ala *in vitro* or phosphorylation of Ser^{431} *in vivo* (Fig. 9) indicates that phosphorylation of Ser^{431} may not regulate the catalytic activity of LKB1 directly. One mechanism by which phosphorylation of Ser^{431} could regulate LKB1 function would be to alter its cellular location or to enable it to interact with a regulatory subunit or a substrate. Ser^{431} lies 2 residues N-terminal to a potential prenylation site (Cys^{433}); and therefore, phosphorylation of Ser^{431} could potentially regulate prenylation of LKB1 *in vivo*. A C-terminal fragment of LKB1 expressed as an N-terminal green fluorescent protein fusion was shown to be prenylated *in vivo*, but it was not demonstrated in this study whether full-length LKB1 was prenylated (10). We have demonstrated here that full-length LKB1, when overexpressed in 293 cells, is farnesylated, but that a mutant form of LKB1 in which the predicted farnesyl acceptor Cys residue is mutated to Ala (LKB1(C433A)) is not prenylated (Figs. 10*B* and 13). As LKB1(S431A) and LKB1(S431D) are prenylated to a similar extent as wild-type LKB1 (Fig. 10*B*), this indicates that phosphorylation of LKB1 at Ser^{431} may not affect prenylation of this enzyme. Unlike Ras, which is prenylated and exists solely at the membranes of cells, subcellular fractionation experiments indicated that only a small fraction of endogenous cellular LKB1 in Rat-2 cells was associated with the membrane fraction (Fig. 10*A*). Furthermore, stimulation of cells with EGF or forskolin to induce phosphorylation of Ser^{431} did not significantly alter the amount of LKB1 present in the membrane fractions of these cells. Although most of the LKB1 was located in the cytosolic fraction of cells, no phosphorylation of cytosolic LKB1 was observed following stimulation of cells with forskolin and EGF. In contrast, these stimuli induced potent Ser^{431} phospho-

rylation of LKB1 associated with cell membranes (Fig. 10). This could be explained if membrane-associated LKB1 was a better substrate for p90^{RSK} and PKA than cytosolic LKB1. It is also possible that both membrane and cytosolic LKB1 are phosphorylated, but cytosolic LKB1 may be more efficiently dephosphorylated by a protein phosphatase than the membrane-associated form of LKB1. Alternatively, cytosolic LKB1 may be associated with another protein that prevents it from becoming phosphorylated at Ser^{431}.

There is considerable evidence that PKA (49, 50) as well as p90^{RSK} (14, 51, 52) could play important roles in regulating proliferation and cell survival. One of the cellular targets that p90^{RSK} and PKA may phosphorylate to protect cells from apoptosis is BAD. This protein, in its dephosphorylated form, interacts with the Bcl family member Bcl-x$_L$ and induces apoptosis of some cells. However, after BAD is phosphorylated at Ser^{112} by p90^{RSK} (53, 54) or at Ser^{155} by PKA (18, 55–57), it dissociates from Bcl-x$_L$ and interacts with 14-3-3 instead, and apoptosis is prevented. However, BAD has a very restricted tissue distribution, suggesting that p90^{RSK} and PKA may arrest the apoptotic pathway or regulate proliferation by phosphorylating additional targets. LKB1 could represent one of these targets, as there is strong evidence that it functions as a tumor suppressor and thus could play a role in regulating cellular transformation. This is based on the findings that many of the mutations identified in LKB1 in patients with Peutz-Jeghers syndrome would be expected to impair its activity (1, 3, 4) and that overexpression of LKB1 in several cancer cells inhibited the proliferation of these cells (5). We have confirmed these findings and demonstrated that mutation of Ser^{431} to either Ala or Asp greatly reduced the ability of LKB1 to suppress the growth of G361 melanoma cells (Fig. 11). As LKB1(S431A) is similar to LKB1(S431D) in this assay, it is possible that mutation of Ser^{431} to Asp is not sufficient to mimic phosphorylation of LKB1 at this residue. It also cannot be discounted that both the phosphorylation and dephosphorylation of Ser^{431} are required for LKB1 to suppress growth. The discovery that LKB1 is a physiological target of p90^{RSK} and PKA is intriguing, and it is tempting to speculate that some of the effects of p90^{RSK} and PKA on cell survival and proliferation could be mediated through phosphorylation of LKB1.

The finding that prenylation of LKB1 is not essential for its ability to suppress the growth of G361 cells (Fig. 12*B*) indicates that farnesylation of LKB1 instead of activation of LKB1 may play a role in inhibiting the function of LKB1 *in vivo*. A mutant of LKB1 that is not prenylated still can phosphorylate itself and p53 to a similar extent as wild-type LKB1 (Fig. 12*B*) and is still phosphorylated at Ser^{431} in cells in response to agonists that activate PKA and p90^{RSK} (Fig. 12*C*). However, as only ~10% of LKB1 that is expressed in 293 cells is prenylated (Fig. 13), it is not possible to conclude whether or not prenylation of LKB1 negatively regulates LKB1 activity, as it would be necessary to obtain a population of LKB1 that was farnesylated to a high stoichiometry to address this question. It is possible that prenylation may regulate the cellular location of LKB1, the stability of LKB1, or its interaction with a regulatory subunit or substrate. In the future, it will be important not only to identify the function of phosphorylation of LKB1 at Ser^{431}, but also to discover the role that farnesylation and/or defarnesylation of Cys^{433} plays in enabling LKB1 to inhibit cell transformation.

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